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Effects of Sangju Honey on Oral Squamous Carcinoma Cells

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Since ancient times, honey has been used in traditional medicine owing to its pharmacological effects. It possesses anticancer properties. However, the therapeutic implications of Sangju honey in cancer remains unknown. Therefore, we aimed to demonstrate the potential anticancer effects of Sangju honey on human oral squamous cell carcinoma (OSCC), particularly focusing on epithelial–mesenchymal transition (EMT) and apoptotic and mitogen-activated protein kinase (MAPK) signaling pathways. Ca9-22 and YD-10B human OSCC cells were treated with 0.25% or 0.5% Sangju honey, and the cell viability was examined using the Cell Counting Kit-8 assay. Cell morphology studies were conducted to observe morphological changes, and the wound-healing assay was performed to evaluate the proliferation of honey-treated OSCC cells. Western blot analysis was conducted to investigate protein expression related to EMT and apoptotic and MAPK signaling pathways. Sangju honey reduced cell viability, induced morphological changes, and significantly suppressed the proliferation and migration of Ca9-22 and YD-10B cells. The expression of E-cadherin and N-cadherin was increased and decreased, respectively, in both OSCC cell lines. Moreover, Sangju honey stimulated apoptosis by increasing the expression of p21, p53, cleaved caspase 3, and caspase 9. Furthermore, it downregulated the expression of phospho (p)-extracellular signal-regulated kinases 1 and 2, p-c-Jun amino-terminal kinase, and p-p38 in Ca9-22 and YD-10B cells. Sangju honey inhibits Ca9-22 and YD-10B cell proliferation by regulating EMT, inducing apoptosis, and suppressing the MAPK signaling pathway. Thus, it is a potential anticancer agent for human OSCC.

Key Words Sangju honey, Oral squamous cell carcinoma, Cell proliferation, Apoptosis, Mitogen-activated protein kinases

INTRODUCTION

Oral cancer is one of the 10 most common cancers in worldwide that ocuurs in oral cavity [1]. Even though oral cancer is diagnosed in early stage, an advanced stage is relatively unpresented by poor prognosis [2]. The 5-year survival rate of nonsurgical patients in squamous cell carcinoma was 44.2%, and for patients who had been supported were still approximate 50% [3]. The conventional treatments of oral cancers include surgery, chemotherapy, and radiation therapy, depending on the diagnosis of individual [4]. Though chemotherapy and radiotherapy are common methods of oral cancer treatment, adverse effect and drug resistance are still severe problems [5].

Many natural products have therapeutic or preventive effects on pathogenesis of cancer and other diseases [6]. Bioactive components in natural products can reduce side effects of chemotherapy and radiotherapy [5].

Honey is one of the most frequently used natural products for therapeutic purposes. Honey has long been used its nutritional and therapeutic advantages [7]. Honey also has been reported to have antioxidant [8], antimicrobial [8], anti-inflammatory [9], antimetastatic [10], and anti-proliferative [11] effects. For instance, Manuka honey is a monofloral honey that

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has been widely investigated due to its biological properties, such as antioxidant and anticancer effects [12,13]. Australia Eucalyptus honey is also popular to consumers due to its medicinal properties [14]. Moreover, several honeys showed anticancer effects [15-18]. However, effects of Sangju honey which is derived from persimmon flower have not been investigated yet.

There are many types of mechanisms to be exploited for cancer therapy. Epithelial-mesenchymal transition (EMT) is a process that is associated with cancer cell migration, invasion, and metastasis [19]. In EMT, cadherin proteins are important to maintain tissue organization and contribute to cell to cell interactions called adherent junctions [20]. Numerous reports indicated that certain natural products regulate EMT in various cancers [21-24]. Some studies also revealed that honey could control cancers via the EMT process [25-27].

Apoptosis is a cellular event of cell death which is also a popular target for cancer treatments [28]. Multiple studies presented that natural products inhibit cancer cell growth via an apoptotic pathway. Honey also induces apoptosis of cancer cells through upregulation of p53, caspase 3 and caspase 9 involved in extrinsic or intrinsic apoptotic pathways [29].

The mitogen-activated protein kinase (MAPK) signaling plays an essential role in cell proliferation [30]. The extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinase (JNK), and p38 families regulate development of cell growth [31]. Previous studies showed that honey could inhibits cancer cell growth via suppression of ERK1/2 and p38 [32,33].

In this study, we investigated effects of Sangju honey on EMT apoptosis and the MAPK signaling in human oral cancer cells. Furthermore, we investigated the underlying mechanisms of Sangju honey in oral cancer.

MATERIALS AND METHODS

Cell lines

Human oral cancer cell lines (Ca9-22 and YD-10B) were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco™; Thermo fisher Scientific, NY, USA) with 10% fetal bovine serum (GenDEPOT, Katy, TX, USA) and 1% penicillin/streptomycin (Gibco™). Cells were incubated at 37˚C in a 5% CO_2 incubator.

Reagents

Sangju honey (Simdanjeong, Sangju, Korea), Australia Eucalyptus honey (Capilano honey Ltd., Richlands, QLD, Australia) and Manuka honey (Arataki Honey Ltd., Waiotapu, Rotorua, New Zealand) were diluted to 1% (w/v) in each culture medium and before each experiment, required concentrations (0.25% or 0.5%) were prepared by serial dilution.

Cell viability assay

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assays were performed to evaluate cell viability. Ca9-22 and YD-10B cells were seeded in 96-well cell culture plates at a density of 2×10^3 cells/well and incubated overnight at 37˚C in a 5% CO_2 incubator. After 12 hours of incubation, 100 μL of the medium containing honey was added to each well of 96-well plates and the cells were cultured for 72 hours. Then, 20 μL of the CCK-8 solution was added to per well and incubated for an additional 2 hours at 37˚C. The absorbance was measured at 450 nm using an Epoch microplate reader (BioTek, St. Louis, CA, USA). Experiments were conducted in triplicate.

Cell morphology

Ca9-22 and YD-10B cells were seeded in 6-well cell culture plates and cultured overnight at 37˚C in a 5% CO_2 incubator. Then, the cultured medium was changed to media with 0.25% or 0.5% honey and maintained for 72 hours in a 5% CO_2 at 37˚C. The cells were examined by an inverted microscope (Leica Biosystems, Seoul, Korea) at 72 hours. All images were captured with Leica imaging system (Leica Biosystems).

Wound healing assay

The wound healing assay was performed to assess oral cancer cell migration. Ca9-22 and YD-10B cells were seeded in 6-well cell culture plates at a density of 8×10^5 cells/well and incubated overnight at 37˚C. After 12 hours, the cell monolayer was scraped in a straight line to create a scratch with a 200 μL pipette tip. Then, cultured medium was discarded and treated with 0.25% or 0.5% honey after PBS washing. Cells were sustained for 6 hours and examined by an inverted microscope (Leica Biosystems) every 3 hours. All images were captured with Leica imaging system (Leica Biosystems).

Western blot assay

Ca9-22 and YD-10B cells were plated in 10-cm cell culture dishes and cultured overnight at 37˚C. Then, cultured medium replaced to media with 0.25% or 0.5% honey and retained for 48 hours at 37˚C in a 5% CO_2 incubator. Then, the cells were harvested and lysed using RIPA II cell lysis buffer (1×) with Triton w/o EDTA with protease inhibitor cocktail (100×). The protein concentrations of the cell lysates were determined with the BCA protein assay kit (Thermo fisher Scientific, Inc., Waltham, IL, USA) and analyzed by an Epoch microplate reader (BioTek). A total of 30 μg of protein was loaded onto 8%–15% by SDS-PAGE and transferred to a PVDF membrane (Merck Millipore, Seoul, Korea). The membranes were blocked in 5% skim milk with 1× TBS-Tween-20 (TBST; including 0.05% Tween-20) for 1 hour and then incubated overnight at 4˚C in primary antibodies diluted with 3% skim milk in 1× TEST. The primary antibodies against phospho-(p)44/42 MAPK (ERK1/2) (Thr202/Tyr204), p44/42

MAPK (ERK1/2), p-Stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185), SAPK/JNK, p38 MAPK, p-p38 MAPK (Thr180/Tyr182), p21 Waf1/Cip1 (12D1), cleaved caspase-3 (D175), and caspase-9 (C9) were purchased from Cell Signaling Technology (Danvers, MA, USA). E-cadherin (67A4), N-cadherin (13A9), p53 (FL-393), and β-actin (C4) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Then, the membranes were washed four times with 1× TBST and incubated with HRP-conjugated secondary antibody with 3% skim milk in 1× TBST (Santa Cruz Biotechnology) for 1 hour at room temperature, followed by three times washing with 1× TBST. Protein expression was detected by the ImageQuant LAS 500 (Cytiva, Incheon, Korea) with an ECL detection kit (Thermo fisher Scientific). The protein expression level of β-actin was used as the internal control.

Statistical analysis

Results are presented as mean ± standard deviation from three independent experiments. Statistical significance was determined by the Student's *t*-test. A *P*-value less than 0.05 showed a statistically significant difference.

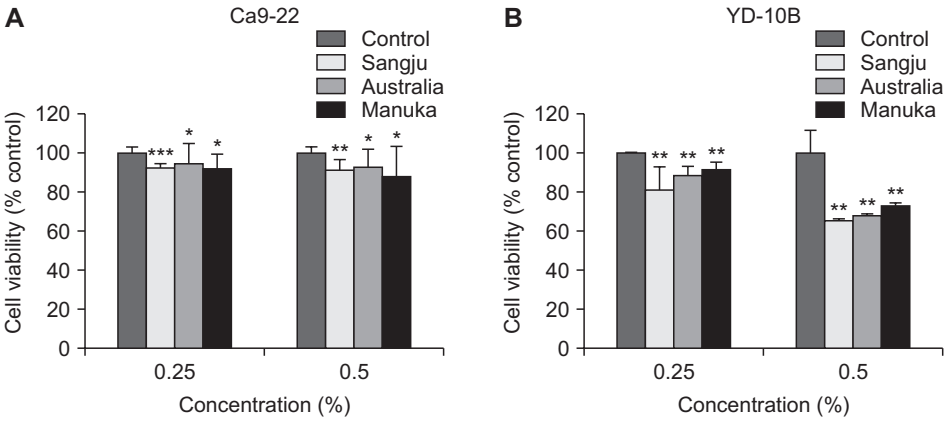

Figure 1. Sangju honey inhibits cell proliferation in oral cancer cells. (A, B) Cell viability examined by the Cell Counting Kit-8 assay. Ca9-22 and YD-10B cells were treated with 0.25% or 0.5% honey for 72 hours. The experiments were conducted in triplicate and shown as the mean ± standard deviation (n = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to control.

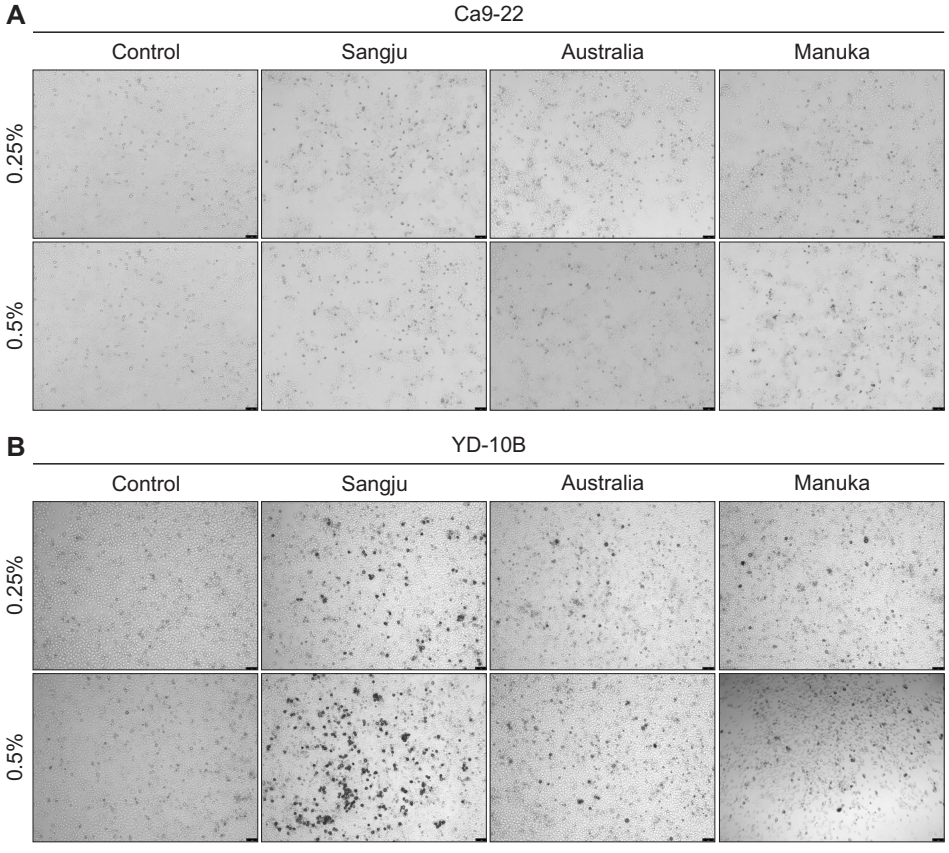

Figure 2. Sangju honey has antiproliferative effect in oral cancer cells. (A) Cell morphology of Ca9-22 treated with 0.25% or 0.5% of honey. (B) Cell morphology of YD-10B exposed with 0.25% or 0.5% of honey. Oral cancer cells were observed with an inverted light microscope at 5 × magnification. Data were representative of three independent experiments.

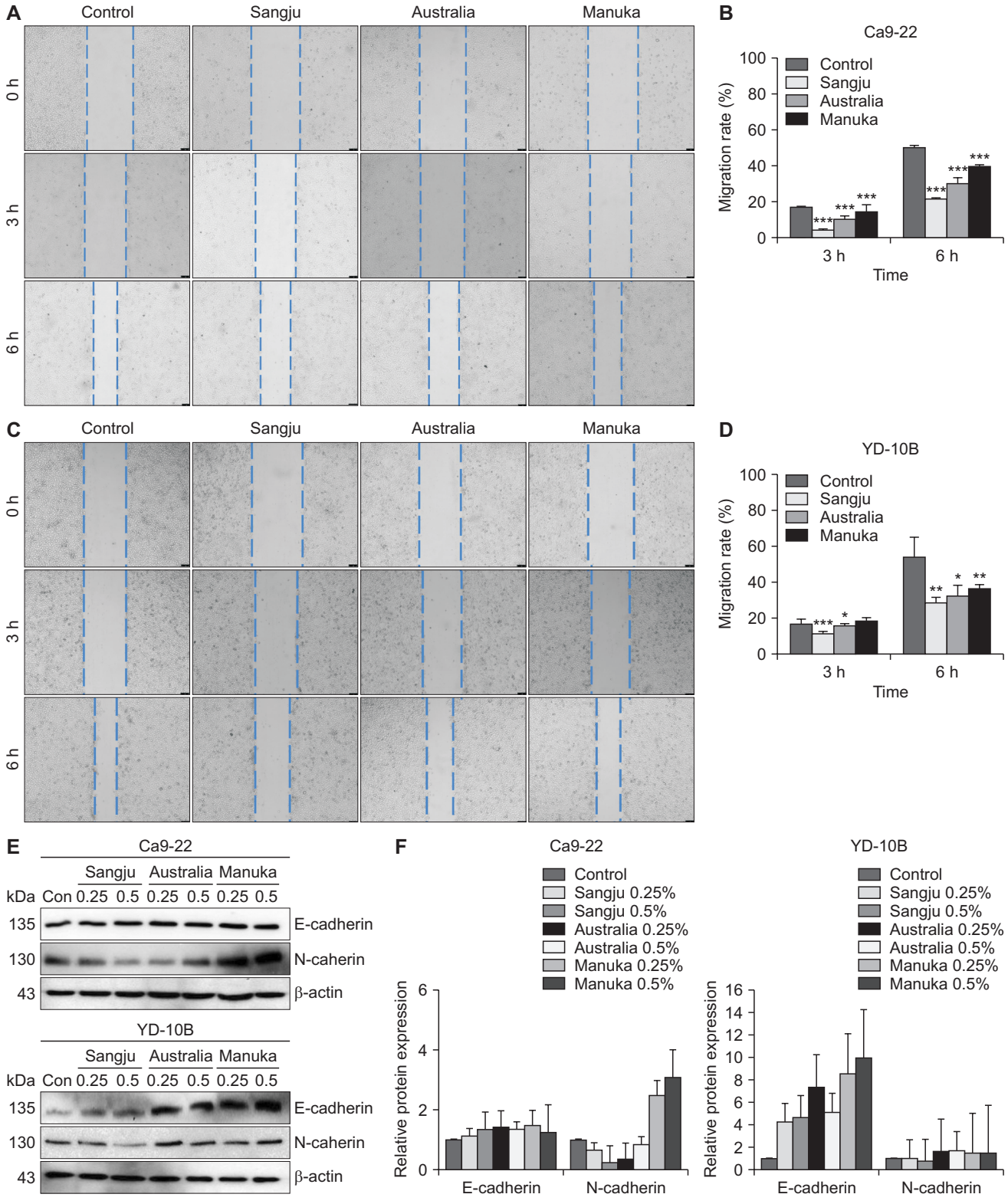

Figure 3. Sangju honey decreases migration abilities of oral cancer cells. (A, B) The migration rate of Ca9-22 cells was measured every 3 hours after treatment with 0.25% or 0.5% of honey. (C, D) The migration rate of YD-10B cells was determined every 3 hours after treatment with 0.25% or 0.5% of honey. Cell migration was observed with a light microscope at 5 × magnification. (E) Western blot analysis on the expression of E-cadherin and N-cadherin in Ca9-22 and YD-10B cells after treated with 0.25% or 0.5% of honey for 48 hours. (F) Relative protein expression levels in Ca9-22 and YD-10B cells were quantified by ImageJ. The experiments were conducted in triplicate and shown as the mean ± standard deviation (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

RESULTS

Sangju honey inhibits cell proliferation in oral cancer cells

To evaluate the viability of honey treated oral cancer cells, the CCK-8 assay was performed on two different oral squamous cell carcinoma (OSCC) cells. Ca9-22 and YD-10B cells were treated with 0.25% or 0.5% honey for 72 hours. As shown in Figure 1A and 1B, Sangju honey inhibited Ca9-22 and YD-10B cells proliferation in a concentration-dependent manner.

Sangju honey has antiproliferative effects in oral cancer cells

To assess morphological changes, Ca9-22 and YD-10B cells were exposed to 0.25% or 0.5% of honey for 72 hours. Ca9-22 cells treated with Sangju honey exhibited reduced cell density, deflation, and round cell morphology (Fig. 2A). Sangju honey also reduced YD-10B cell density, caused shrinkage, and changed the cell morphology to be circular (Fig. 2B).

Sangju honey represses the migration capabilities of oral cancer cells

Then, we analyzed whether Sangju honey could diminish oral cancer cell migration. The migration of Ca9-22 and YD-10B cells was remarkably inhibited by Sangju honey in concentration and time-dependent manners (Fig. 3A-3D). The EMT process is closely associated with cancer invasion, proliferation, and metastasis that can make tumors more malignant by potentiating their metastatic activity [20]. Therefore, to evaluate the effect of Sangju honey on proliferation and metastasis of oral cancer cells, the expression of the EMT marker proteins was investigated. The results indicated that Sangju honey up-regulated E-cadherin and down-regulated N-cadherin in Ca9-22 and YD-10B oral cancer cells (Fig. 3E).

Sangju honey induces apoptosis and inhibits cell growth via MAPK signaling in oral cancer cells

Apoptosis is one of the cell death mechanisms that occurs in physiological and pathological conditions [28]. Accordingly, many anti-cancer therapies are intended to induce cancer cell apoptosis by modulating different steps of the apoptotic

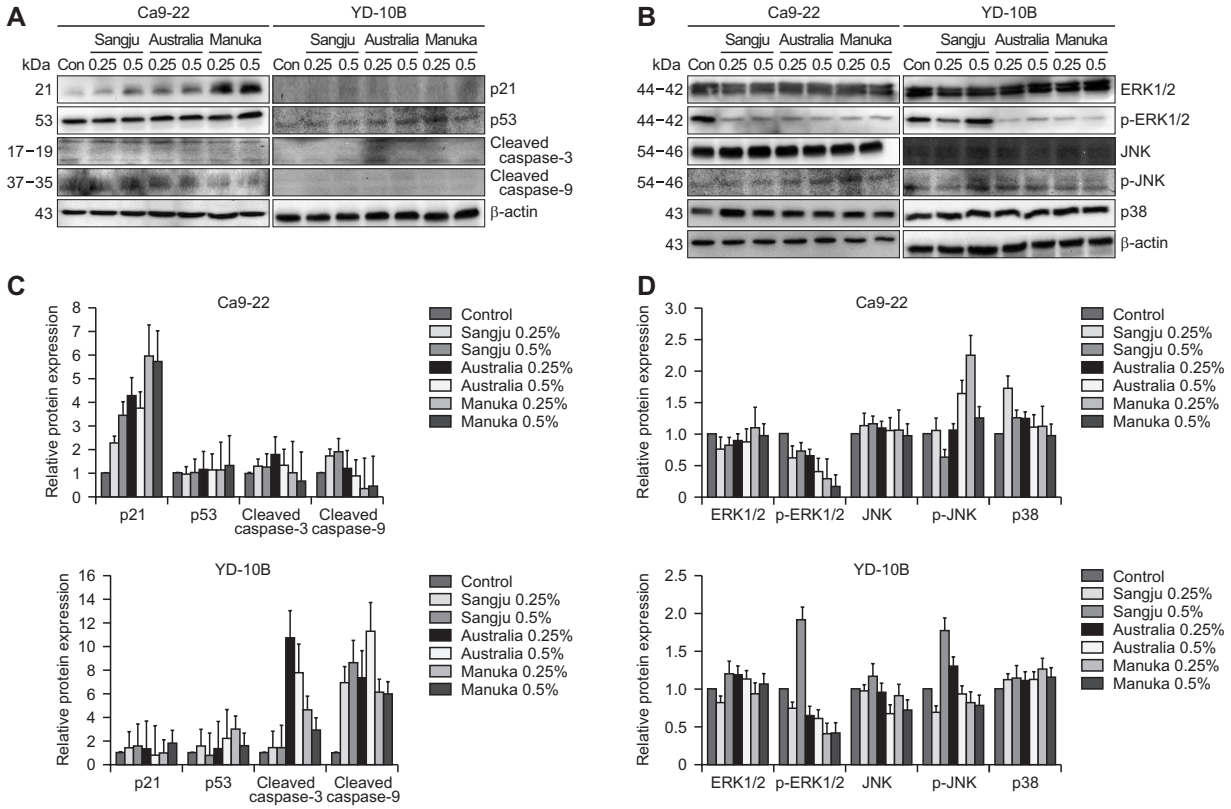

Figure 4. Sangju honey induces apoptosis and inhibits MAPK signaling in oral cancer cells. (A) The expression of the apoptotic marker proteins in Ca9-22 cells after treated with 0.25% or 0.5% honey for 48 hours. (B) The expression of proteins involved in the MAPK signaling pathway in Ca9-22 after treated with 0.25% or 0.5% honey for 48 hours. The protein expression levels in oral cancer cells were quantified by the ImageQuant LAS 500 cells. (C, D) Relative protein expression levels in Ca9-22 and YD-10B cells were quantified by ImageJ. ERK1/2, extracellular signal-regulated kinases 1 and 2; p-ERK, phospho-ERK; JNK, c-Jun amino-terminal kinase; p-JNK, phospho-JNK, p-p38, phospho-p38; MAPK, mitogen-activated protein kinase.

signaling pathway to get rid of malignant tumor cells [34]. To investigate the effects of Sangju honey on apoptosis, Ca9-22 and YD-10B cells were treated with 0.25% or 0.5% of honey for 48 hours. The results showed that Sangju honey induced apoptosis in oral cancer cells by up-regulating p21, p53, cleaved–caspase 3, and cleaved-caspase 9 expressions in Ca9-22 and YD-10B cells (Fig. 4A). The MAPK signaling pathway includes some major signaling components and plays a key role in tumorigenesis, and hence regarded as potential therapeutic target for cancer treatment [35]. As shown in Figure 4B, the expression of p-ERK1/2, p-JNK, and p-p38 associated with MAPK signaling was downregulated in Ca9-22 and YD-10B cells.

DISCUSSION

Natural products have potential for cancer treatment as well as cancer prevention [36]. Several studies demonstrated that honey could regulate growth and progression of diverse types of cancer cells by inhibiting metastasis and cancer-associated signaling pathways [15,16,33,37,38]. However, in OSCC, only few studies described anticancer effects of honey. In this study, we attempted to investigate how Sangju honey regulates cell growth, and elucidate the underlying molecular mechanisms potential mechanisms in OSCC.

Since anticancer effects of Sangju honey have not been demonstrated, we first compared its anticancer potency with other honey samples. First, we chose 10 different types of honey including Sangju honey. Next, oral cancer YD-10B cells were seeded in 96-well cell culture plates and treated with 1.25%, 2.5%, 5% (w/v) of each honey sample respectively for 72 hours. Then, after 72 hours, CCK-8 was performed to examine cell viability. The result demonstrated that both Australia honey and Manuka honey had the highest effects among 10 samples tested (Fig. S1). Accordingly, Australia honey and Manuka honey were chosen for comparison with Sangju honey for an anticancer activity. Reduction of cell viability under 80% is regarded as cytotoxicity, and under 40% of cell viability is considered as strong cytotoxicity [39]. The Australia honey and Manuka honey presented under 40% of cell viability in YD-10B cells. Moreover, Sangju honey also revealed considerable cytotoxicity in a concentration-dependent manner.

Scratch wound healing assay was conducted in OSCC. Cell migration is an inevitable process associated with cancer metastasis [40]. After Ca9-22 and YD-10B cells were seeded in 6-well cell culture plates, we scratched the bottom surface of each well using a pipette tip to create a gap in the monolayer of OSCC cells. Since Ca9-22 and YD-10B cells were seeded at a density of 8×10^5 cells/well, a density of cells was closed to 100% after incubated overnight. For that reason, we measured a gap until 6 hours. The gap sizes of each 3 hours were analyzed by an ImageJ (https://ij.imjoy.io) [41].

Antiproliferative and antimigrative effects of Sangju honey were investigated in OSCC at a molecular level by using western blot assay. First, we examined expression of proteins related to cell migration, metastasis, and cell proliferation based on results from cell viability and wound healing assays. EMT is closely connected to cancer that can promote the motility and metastatic activity of solid tumor cells [21]. We analyzed E-cadherin and N-cadherin which differentially modulate proliferation of adherent cells as adherens junction proteins in OSCC [20]. In human cancer, normally E-cadherin expression is down-regulated, whilst N-cadherin is up-regulated [20]. The result showed that Sangju honey elevated the E-cadherin level and strongly down-regulated N-cadherin expression compared to Australia and Manuka honey in Ca9-22 and YD-10B cells.

Next, since Sangju honey suppressed cell migration, metastasis, and cell proliferation in OSCC, we also investigated apoptotic pathway and MAPK signaling pathway mechanisms that are linked to cancer cell growth. p53 is known as a tumor suppressor protein. In term of apoptotic mechanism, down-regulated p53 causes reduction of apoptosis and increases tumor growth and development [42]. Therefore, we examined expression of p53 in OSCC. We also assessed expression of p21, which is downstream effector molecule of p53 [43]. The expression of p21 in Ca9-22 and YD-10B cells treated with Sangju honey was upregulated compared to the untreated control group. This result indicated that Sangju honey could induce apoptosis and suppress OSCC growth. Moreover, we particularly focused on the intrinsic pathway of apoptosis. In the intrinsic pathway, cytochrome *c* which is released to cytoplasm from mitochondria activates caspase 3 through activation with caspase 9 [28]. Taken together, Sangju honey could regulate p53, p21, caspase 9, and caspase 3 in inducing OSCC apoptosis apoptotic pathway.

MAPK signaling associated with cell proliferation is activated through phosphorylation of its component kinases [44]. Therefore, we examined active forms of ERK, JNK, and p38 which are p-ERK, p-JNK, and p-p38. Sangju honey inhibits

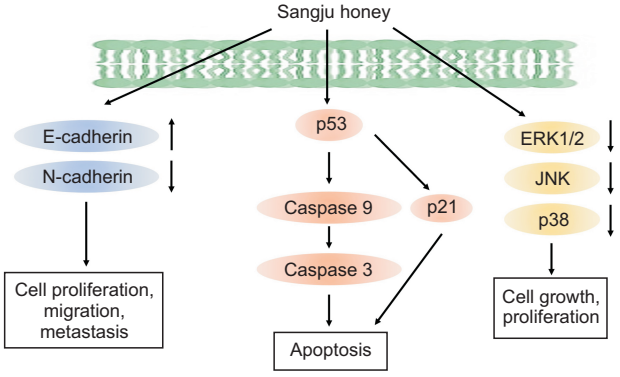

Figure 5. Proposed mechanisms underlying inhibition of oral cancer cell growth by Sangju honey. ERK1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun amino-terminal kinase.

cell growth by downregulating p-ERK, p-JNK, and p-p38 expression in Ca9-22 and YD-10B cells. Taken together, our results demonstrate that Sangju honey significantly inhibits proliferation, migration, metastasis, growth of OSCC via the EMT, intrinsic apoptotic pathway, and the MAPK signaling. Further studies will be necessary to verify the anticancer effects of Sangju honey in vivo (Fig. 5).

FUNDING

None.

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2022.27.4.239.

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